

Hepato-Protective and Antioxidant Effects of *Spondias Mombin* Leaf and Stem Extracts upon Carbon Tetrachloride-Induced Hepatotoxicity and Oxidative Stress

Lucky L Nwidi¹, Ekramy Elmorsy², Oboma I Yibala³, Wayne G Carter⁴,

¹Department of Experimental Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Port Harcourt, Choba, East West Road, Rivers State, Nigeria. ²Department of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Mansoura University, Egypt. ³Department of Medical laboratory Science, Faculty of Basic Medical Sciences, College Health Sciences, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria. ⁴School of Medicine, University of Nottingham, Royal Derby Hospital Centre, Derby, UK.

ABSTRACT

Objectives: *Spondias mombin* is used in folk medicine in Nigeria for the treatment of hepatitis. This study comparatively evaluates the *in vivo* hepato-protective and antioxidant effects of *Spondias mombin* leaf (SML) and *Spondias mombin* stem (SMS) methanolic extracts in a rat model of hepatotoxicity. **Methods:** 42 rats were equally divided into seven groups of six animals each. Group A received water, Group B water, Groups C and D received SML at 500 and 1000 mg/kg bw, respectively, Groups E and F received SMS 500 and 1000 mg/kg bw, respectively, and Group G received silymarin at 100 mg/kg. All extracts and drugs were administered daily by oral gavage for a total of seven days, and then for Groups B to G acute hepatotoxicity was induced by administering CCl₄. After 48 hours rats were sacrificed and assayed for histological and biochemical indices of hepatotoxicity. **Results:** CCl₄ treatment induced liver injury, with significantly increased levels of markers of hepatocellular injury alanine aminotransferase (ALT), aspartate transaminase (AST) total bilirubin (TBIL) and conjugated bilirubin (CBIL), as well as a significant reduction of total circulatory protein. SML or SMS plant extracts at 500 and 1000 mg/kg prior to CCl₄ treatment significantly ameliorated liver injury, and decreased the levels of ALT, AST, TBIL, and CBIL. SML or SMS extracts significantly increased cellular levels of glutathione, the

activities of catalase and superoxide dismutase, and significantly decreased thiobarbituric acid reactive substances. **Conclusion:** This study provides preliminary evidence supporting the potential benefit of *Spondias mombin* for treatment of xenobiotic-induced hepatotoxicity.

Key words: Antioxidant, hepato-protection, hepatotoxicity, oxidative stress, *spondias mombin*

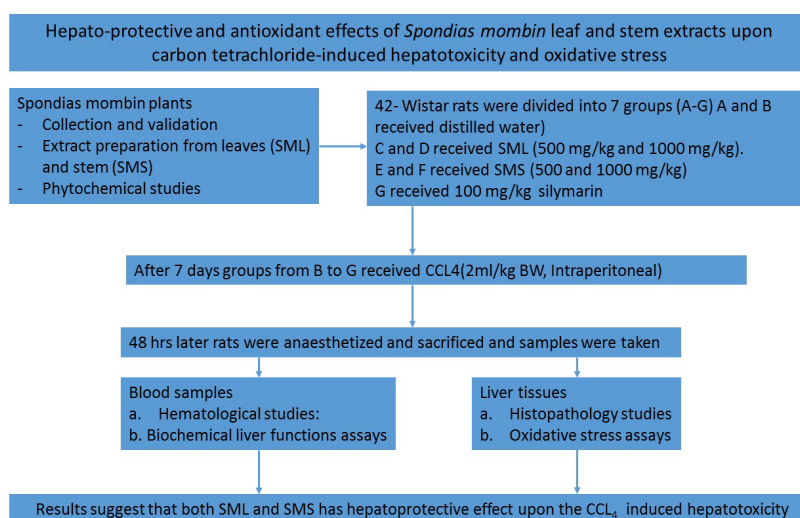
Correspondence:

Lucky Nwidi,
Department of Pharmacology, Faculty of
Pharmacy, Niger Delta University, Yenegoa,
Nigeria.
E-mail: menelucky@yahoo.com

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INTRODUCTION

Liver diseases contribute markedly to the global burden of diseases and are major causes of illness and death worldwide.^[1-4] Liver diseases remain a public health challenge, for which the development of new pharmaceutical treatments are required. The evaluation of the hepato-protective benefits of medicinal plants using laboratory animals is a useful initial step in determining drug safety of new biomolecules.^[5-9] Natural products from ethnomedicine provide safe and effective alternative treatments for hepatotoxicity. Many previous reports have associated these hepato-protective effects with endogenous phytoextracts or phyto-compounds that are rich in natural antioxidants.^[5-15] Hence an increasing number of bioactive compounds and plant extracts have been evaluated for hepato-protective and antioxidant

effects against hepatotoxin-induced liver damage.^[16] The phenolic compounds commonly found in both edible and traditional medicinal plants are incriminated with multiple biological activities, including

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free radical scavenging activity.^[18-20] It has been suggested that natural antioxidants in food, such as phenolic compounds or flavonoids, might play an essential role in the prevention of oxidative stress-related disorders and diseases, and in the reduction of premature mortality.^[21-22] Flavonoids are certainly ubiquitous in the epidermal cells of plant parts such as the flowers, leaves, stems, roots, seeds, and fruits, and exist in glycosidic and non-glycosidic forms.^[23]

Spondias mombin L. (Anacardiaceae) is commonly known as (English), akika (Yoruba), ijikara (Igbo), tsadarmaser (Hausa), chabbuh (Fulani), nsukakara (Efik) and "atoa" (Ashanti).^[24] It is a deciduous erect tree, which grows up to 15-20 meters in height with a trunk 60-75 cm wide.^[25-26] *Spondias mombin* is commonly found in the tropical Americas, including the West Indies, and has been naturalized in parts of Africa, including Ghana, and some parts of Asia.^[26] In ethnomedicine, *Spondias mombin* plant parts including the stem-bark, leaves, and roots have been used for the treatment of various conditions. *Spondias mombin* possesses anti-microbial^[27,28], and anti-viral activities^[29], with leaves used, for example, for their anti-inflammatory^[30], anthelmintic activity^[31], haematinic^[32], and sedative^[33] activities, and stem-bark has an anti-mycobacterial^[34] activity. Phytochemical screening indicates that the *Spondias mombin* leaf (SML) contains tannins, saponins, alkaloids, flavonoids and phenols.^[35] The leaves are also rich in ascorbic acid, niacin, and contain riboflavin and thiamine.^[35] Although the hepatoprotective effects of SML and *Ocimum gratissimum* have been evaluated in rats after intoxication with dimethyl nitrosamine,^[36] the effects of SML or SMS on CCl₄-induced hepatotoxicity has yet to be assessed. Hence the aim of this study was to establish if SML and SMS methanolic extracts are hepato-protective to CCl₄-induced hepatotoxicity in rats.

MATERIALS AND METHODS

General reagents and chemicals

Carbon tetrachloride, silymarin, diethyl ether and methanol were purchased from Sigma-Aldrich, St. Louis, Missouri, USA. Randox Diagnostic kits for serum alanine aminotransferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), conjugated bilirubin (CBIL) and total bilirubin (TBIL) were purchased from Randox Laboratories Ltd. London, UK. Other chemicals and solvents were of the highest (analytical) grade commercially available and obtained from either Sigma-Aldrich or Merck, UK.

Collection and plant validation

Fresh leaves and stem of *Spondias mombin* L. were collected from Obafemi Awolowo University campus during the month of January, 2015. The plant was identified and authenticated by Dr. Oladele Adekunle, a Taxonomist with the Forestry Department of the University of Port Harcourt. A specimen of SML (20015) and SMS (20016) were deposited at Forestry Department of the University of Port Harcourt, Nigeria.

Preparation of plant extract

Fresh leaves and stem-bark of *Spondias mombin* L. were weighed, air dried and powdered. The powdered leaves (300g) and powdered stem (300g) were extracted using a cold extraction method (maceration) using methanol as solvent. The SML and SMS powder were soaked in one litre of 50% methanol for a period of 72 hours during which the mixture was shaken twice daily to promote extraction. The solvent was filtered over a layer of gauze and then the filtrate evaporated to dryness *in vacuo* at 55°C. The weight of the dried leaf extract was 21.3g, and for

the stem, 9.4g. The yield obtained was 7.1% and 3.1% for SML and SMS extracts, respectively. The extract was stored in a refrigerator for up to four weeks for use in assays.

Phytochemical screening

The SML and SMS extracts were quantitatively assayed for the presence of phytochemicals such as saponins, tannins, alkaloids, terpenoids, cardiac glycosides and flavonoids using the following standard procedures.

To detect reducing sugars

Fehling's Test: Fehling's solution A (1 mL) and Fehling's solution B (1 mL) were mixed with 1 mL of SML or 2 mL of SMS and heated in a boiling water bath for 10 minutes. The appearance of yellow and then brick red precipitate was indicative of the presence of reducing sugars.

Benedict's test: An equal volume (2 mL) of Benedict's solution and SML or SMS extracts were mixed in a test tube and heated in a boiling water bath for 10 minutes. The changes in colour to yellow, green and red reflected the presence of reducing sugars.

To detect alkaloids: 10g of each of the dry extracts were mixed with 20 mL of dilute hydrochloric acid. The mixture was shaken well and then filtered. The filtrate was used for the following tests.

Mayer's test: To 3 mL of the filtrates, 1 mL of Mayer's reagent (potassium mercuric iodide) was added. The appearance of a white precipitate was indicative of the presence of alkaloids.

Wagner's test: To 3 mL of the filtrates, 1 mL of Wagner's reagent (iodine in potassium iodide) was added. The appearance of a reddish brown precipitate indicated the presence of alkaloids.

Dragendorff's test: To 3 mL of the filtrates, 1 mL of Dragendorff's reagent (potassium bismuth iodide) was added. The appearance of a brick red precipitate showed the presence of alkaloids.

To detect glycosides

Borntrager's test: To test tubes containing 2 mL of either extract, 2 mL of dilute sulphuric acid was added, and the mixture boiled for 5 min and then filtered. To the filtrate, an equal volume of chloroform was added and mixed. The organic layer was separated and then ammonia added. The presence of a pinkish red colour within the ammonia layer was indicative of the presence of anthraquinone glycosides.

Keller-killiani test: To test tubes containing 2 mL of either extract, 1 mL of glacial acetic acid, 3 drops of 5% (w/v) ferric chloride, and concentrated sulphuric acid were added. The disappearance of the reddish-brown colour at the junction of the two layers, and bluish-green colouration within the upper layer was consistent with the presence of cardiac glycosides.

To detect flavonoids

Shinoda test: To either of the dry extracts (2g), 5 mL of ethanol (95% v/v), 5 drops of hydrochloric acid and 0.5g of magnesium turnings were added. The generation of a pink colouration to the solution suggested the presence of flavonoids.

To detect saponins

Foam Test: To 2g of either extract 20 mL of water was added and the mixture shaken vigorously and observed for persistent foaming, indicative of the presence of saponins.

To detect tannins and phenolics

Ferric chloride test: To 3 mL of either extract, 3 mL of 5% (w/v) ferric chloride solution was added. Formation of a blue-black colour

suggested the presence of tannins and phenols.

Lead acetate test: To 3 mL of either extract, 3 mL of lead acetate solution was added. The generation of a white precipitate indicated tannins and phenols were present.

Experimental animals

Forty-two healthy Wistar rats of average weight (320-355 g) of either sex (21 male, 21 female) were purchased from the Animal House of the Department of Pharmacology, Faculty of Pharmacy, Niger Delta University, Bayelsa State. The animals were acclimatized for one week prior to experimentation. All the animals were fed on a standard chow diet and given access to water *ad libitum*. Experimental techniques and protocols used in this study follow the "Guide to the care and use of animals in research and teaching"^[37] as adopted and approved by Niger Delta University, Bayelsa State, Nigeria, Institutional Animal Care and Use Committee (NDUAEC) on 20/02/2015 via an approved circular No. NDU/2014/007.

Acute toxicity study

To ascertain an approximate LD₅₀ value for a small mammal, Albino mice (25-30g) of either sex were used. Animals were divided into eight groups of three animals per group. Doses of 100, 500, 1000, 2000, 3000, 4000, and 5000 mg/kg were administered intraperitoneally per animal. The treated animals were monitored for 24 hours for mortality and behavioural changes consistent with toxicity.^[38,39]

Experimental design

A total of 42 animals were weighed and divided into seven groups of six animals each (3 male, 3 female). The protocol used for induction of hepatotoxicity was as follows: Group A (normal control): received distilled water (0.2 ml/kg bw by oral dosing), Group B received distilled water (0.2 ml/kg bw by oral dosing), Groups C and D received SML at 500 and 1000 mg/kg bw, respectively, dissolved in distilled water, Groups E and F received SMS 500 and 1000 mg/kg bw, respectively, dissolved in distilled water, and Group G received silymarin as a 100 mg/kg suspension in distilled water. All of the extracts and drugs were administered daily by oral gavage for a total of seven days. On the seventh day, Groups B to G were treated with a mixture of freshly prepared CCl₄ in liquid paraffin (2ml/kg bw, 1:1 intraperitoneally) one hour after administration of the last dosing. Body weights of all rats were recorded daily throughout the seven days of treatment.

After 48 hours rats were anesthetized using diethyl ether prior to sacrifice. Blood was obtained by cardiac puncture into an EDTA vacutainer for determination of hematological parameters of the blood samples using an Automated Hematological Analyzer, SYSMEX – KX21 (SYSMEX Corporation, Japan). The hemoglobin concentration (Hb), packed cell volume (PCV), red blood cell count (RBC), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), white blood cell count (WBC), and platelet count (PLC) were determined.

For biochemical assessment, blood was spun at 3000 rpm for 10 minutes at 4°C to separate serum into vacutainer vials and stored at 4°C until used for analyses. Livers were immediately extracted and perfused with ice-cold saline (0.9% sodium chloride) before utilisation for further analyses.

Measurement of biochemical parameters

The serum collected was used to determine Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaline Phosphatase (ALP), conjugated bilirubin (CBIL), total bilirubin (TBIL) and total protein (TP) using Randox diagnostic kits. These analyses were performed at the Department of Chemical Pathology, Niger

Delta Teaching Hospital (NDUTH), Okolobiri, Bayelsa state, Nigeria.

Measurement of hepatic antioxidant enzymes

Liver tissues from experimental animals were perfused with ice-cold saline and transported from Niger Delta University, Faculty of Pharmacy Pharmacology Laboratory on dry ice to the School of Medicine, University of Nottingham, Royal Derby Hospital Centre, Derby, UK and stored at -80°C until required. Liver pieces (100 mg) were diced and homogenized in 100 mL of 5 mM Tris/HCl buffer (pH 7.4), 1 mM EDTA and complete mini protease inhibitor cocktail (Roche). Homogenates were then centrifuged at 10,000 rpm at 4°C for 10 minutes and the clear supernatant used for the estimation of antioxidant parameters (glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and also measurements of thiobarbituric reacting substances (TBARS).

Glutathione levels

GSH levels were determined based on the published method of Ellman *et al.*^[40] Homogenate (0.2 mL) was mixed with 25% trichloroacetic acid (TCA) and centrifuged at 3000 rpm for 10 min. The supernatant (~0.2 mL) was mixed with 10 mM of DTNB in the presence of phosphate buffer (0.1 M, pH 7.4) and the absorbance read at 420 nm.

Catalase activity measurements

The determination of catalase assay was performed based upon the method of Aebi.^[41] The assay relies upon the ultraviolet absorption of hydrogen peroxide that can be measured at 240 nm. Assays were performed in the presence of 50 mM phosphate buffer. Hydrogen peroxide decomposition was monitored in a 96 well Quartz plate using a Spectramax (Thermofisher) microplate reader. Catalase activity was expressed as units/mg protein.

Superoxide dismutase (SOD) activity measurements

Liver cytosolic SOD activity was measured according to the method of Kakkar *et al.*^[42] Cytosol (0.05 mL) was mixed with sodium pyrophosphate buffer (0.052 M, pH 8.3, 1.2 mL), phenazine methosulphate (0.186 mM, 0.1 mL), nitroblue tetrazolium chloride (0.3 mM, 0.3 mL), and NADH (0.78 mM, 0.2 mL). The reaction was stopped after 90s by the addition of glacial acetic acid. Colour intensity of the chromogen was extracted in butanol (2.0 mL) with vigorous shaking. The mixture was then centrifuged at 3000 rpm for 10 min and the supernatant extracted and the absorbance at 560 nm determined using a Spectramax microplate reader.

Determination of thiobarbituric reactive substances (TBARS)

Lipid peroxidation was determined spectrophotometrically by measuring the level of lipid peroxidation product, malondialdehyde (MDA), as described by Draper and Hadley.^[43] MDA reacts with

Table 1: Phytochemical constituent of SML and SMS Extracts

Phytochemicals	Observations	EXTRACT	
		SML Extract	SMS Extract
Reducing sugars	Reddish brown precipitate upon heating	+	+
Cardiac glycosides	Brick red precipitate	+	+
Saponins	Persistent froth unbroken upon standing	++	+++
Tannins	Blue black precipitate	++	+++
Flavonoid	Resultant solution turns yellow	+++	+++

(+) to (+++)=detected in moderate to abundant quantities.

Nwidu L: Hepatoprotective Effects of Hydromethanolic Leaf and Stem Extracts of *Spondias mombin* in Carbon Tetrachloride Induced-Hepatotoxicity and Oxidative Stress

thioibarbituric acid (TBA) to form a red/pink coloured complex that absorbs maximally in acid solution at 532 nm. Spectrophotometric measurements were recorded at 532 nm using a Spectramax microplate reader.

Histopathological studies

Portions of rat liver from each rat of each group were cut into pieces of approximately 6 mm³ size and fixed in phosphate buffered 10% formaldehyde solution. Liver pieces were embedded in paraffin wax before thin sections of 5 µm thickness cut and then stained with hematoxylin-eosin (H and E). These thin liver sections were made into permanent slides and examined using a high-resolution microscope after which photomicrographs were taken.

Statistical analysis

All statistical measures were performed using PRISM 5 (GraphPad Software Inc., San Diego, California USA). Unless specified otherwise

results are expressed as means ± standard error of mean (SEM). One way analysis of variance (ANOVA) was used to compare group data, followed by Tukey's multiple comparisons test. A *p* value of <0.05 was considered significant.

RESULTS AND DISCUSSION

Phytochemical studies

Preliminary phytochemical screening of the SML and SMS extract revealed the presence of alkaloids, reducing sugars, saponins, and tannins in both extracts [Table 1]. The SMS extract contained visually more phytochemicals with respect to saponins and tannins than the SML extract, but for other qualitative assays, both extracts were similar.

Acute toxicity study

No lethality was observed in mice after a single dose (p.o.) of either SML or SMS (100-5000 mg/kg bw). From acute toxicity testing, no

Table 2: Effect of *Spondias mombin* (leaf) and (stem) on hematological parameters

PARAMETERS	GROUP A	GROUP B	GROUP C	GROUP D	GROUP E	GROUP F	GROUP G
PCV	42 ± 3.2	49 ± 7.9	44 ± 3.5 ^{ns}	42 ± 3.9 ^{ns}	40 ± 6.2 ^{ns}	44 ± 6.5 ^{ns}	49 ± 3.1 ^{ns}
HB	12 ± 0.6	14 ± 2.1	13 ± 1.8 ^{ns}	11 ± 0.7 ^{ns}	12 ± 0.8 ^{ns}	13 ± 2.3 ^{ns}	13 ± 0.6 ^{ns}
WBC	13 ± 4.9	7 ± 4.2	9 ± 0.4 ^{ns}	11 ± 7.7 ^{ns}	11 ± 4.2 ^{ns}	8 ± 2.3 ^{ns}	13 ± 4.5 ^{ns}
PLT	468 ± 280	459 ± 366	761 ± 1.4 ^{ns}	284 ± 316 ^{ns}	500 ± 288 ^{ns}	446 ± 306 ^{ns}	642 ± 322 ^{ns}
RBC	7 ± 1.3	7 ± 1.2	6 ± 0.0 ^{ns}	6 ± 1.1 ^{ns}	7 ± 0.8 ^{ns}	7 ± 0.9 ^{ns}	8 ± 0.3 ^{ns}
MCV	63 ± 2.5	65 ± 2.0	65 ± 7.1 ^{ns}	66 ± 4.4 ^{ns}	62 ± 2.6 ^{ns}	63 ± 0.8 ^{ns}	65 ± 4.2 ^{ns}
MCH	17 ± 2.0	18 ± 0.3	18 ± 1.7	18 ± 0.5 ^{ns}	17 ± 0.7 ^{ns}	19 ± 1.4 ^{ns}	18 ± 1.2 ^{ns}
MCHC	27 ± 2.7	28 ± 0.5	28 ± 0.4	27 ± 2.1 ^{ns}	26 ± 2.1 ^{ns}	30 ± 2.5 ^{ns}	27 ± 2.1 ^{ns}
NEU	32 ± 8.2	44 ± 13	37 ± 7.1	40 ± 14 ^{ns}	52 ± 13 ^{ns}	39 ± 7.2 ^{ns}	38 ± 5.4 ^{ns}
LYM	63 ± 8.0	44 ± 13	57 ± 9.9	51 ± 15 ^{ns}	42 ± 14 ^{ns}	54 ± 9.3 ^{ns}	54 ± 7.3 ^{ns}
MEB	6 ± 2.4	10 ± 4.1	6 ± 2.1	9 ± 2.1 ^{ns}	6 ± 3.5 ^{ns}	8 ± 3.1 ^{ns}	7 ± 4.3 ^{ns}

Values represent mean ± Standard Error of mean (S.E.M) n=6. Results are displayed relative to positive control values and ^{ns}p>0.05; Statistical analysis was done using one way ANOVA. Group A received 0.2 ml/kg distil water; B received CCl₄ 1ml/kg; C and D received 500 mg/kg and 1000 mg/kg of SML+1 ml CCl₄ each respectively; E and F received 500 and 1000 mg/kg of SMS+1ml CCl₄ each respectively; G received 100 mg/kg of silymarin. Monocytes, eosinophil and basophils (MEB).

Table 3: Effect of *Spondias mombin* (leaf) and *Spondias mombin* (stem) on biochemical parameters

Treatment	Dose mg/kg body weight	TP	ALT (U/L)	AST (U/L)	ALP (U/L)	CBIL (µmol/L)	TBIL (µmol/L)
Control Healthy	0.0	35.40 ± 0.5	45.4 ± 0.5	53.2 ± 0.5	44.8 ± 0.7	0.2 ± 0.08	5.2 ± 0.08
Control Positive	1ml/kg CCl ₄	17.2 ± 0.6 ^c	97.2 ± 0.6 ^c	84.8 ± 2.5 ^c	88.2 ± 0.6 ^c	1.9 ± 0.02 ^c	10.5 ± 0.2 ^c
<i>S. mombin</i> (leaf) SML	CCL ₄ + 500	18.8 ± 0.9 ^{ns}	81.4 ± 2.5 ^a	77.4 ± 2.0 ^{ns}	48.2 ± 2.5 ^a	1.8 ± 0.2 ^{ns}	9.8 ± 0.4 ^{ns}
	CCL ₄ + 1000	33.0 ± 0.6 ^c	53.0 ± 0.6 ^c	53.0 ± 0.6 ^c	45.6 ± 1.5 ^c	0.2 ± 0.08 ^c	5.0 ± 0.1 ^c
<i>S. mombin</i> (stem) SMS	CCL ₄ + 500	22.4 ± 0.7 ^c	78.6 ± 7.7 ^c	78.6 ± 2.2 ^{ns}	52.4 ± 1.5 ^{ns}	1.4 ± 0.2 ^{ns}	9.6 ± 0.5 ^{ns}
	CCL ₄ + 1000	35.2 ± 0.8 ^c	45.2 ± 0.8 ^c	52.0 ± 1.1 ^c	44.4 ± 1.1 ^c	0.3 ± 0.07 ^c	4.9 ± 0.2 ^c
Silymarin	CCL ₄ + 100	34.0 ± 0.5 ^c	46.0 ± 0.9 ^c	21.2 ± 2.1 ^c	44.4 ± 1.6 ^c	0.2 ± 0.05 ^c	5.2 ± 0.05 ^c

Values represent mean ± Standard Error of mean (S.E.M) n=6; Significant results of extracts and pure drug are displayed relative to positive control values; and positive control displayed relative health control; results with significant changes from controls marked with alphabets (a, b, c). For significance: ^ap<0.05; ^bp<0.01 and ^cp<0.001. Statistical analysis was done using one-way ANOVA. TP: Total protein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; CB: conjugated proteins; TB: Total bilirubin. Figure in bracket represents the calculated percentage hepatoprotection.

sign of toxicity was observed 24-72 hours after extract administration. Consequently, we adopted a dose range of 1/10th and 1/5th of the maximal dose examined (5000 mg/kg bw) for both extracts for bioassays.

Weight evaluation

The effect of CCl₄, SML and SMS on the body weights of rats throughout the experimental course is presented in Figure 1. There were significant changes in the weight of the rats of each group through the time course of the experiment (0-7 days) (two-way ANOVA, $p < 0.0001$). However, when considering the effect of treatment, there were no significant differences in body weight among the studied groups when compared to the CCl₄ treated group ($p = 0.506$). There was a 3.5% increase in body weight with 500 mg/kg SMS, and this increased to 4.3% at 1000 mg/kg.

Effect of SML and SMS on haematological parameters

The effects of SML and SMS on hematological parameters are shown in Table 2. Extracts at either 500 or 1000 mg/kg did not have any significant effect on the hematological indices evaluated except SMS at 500 mg/kg which induced a significant ($p < 0.05$) change in PCV when compared to the CCl₄ intoxicated group.

Effect of SML and SMS on hepatic biomarkers

To assess the hepato-protective effect of pre-treatment with SML (500, 1000 mg/kg) and SMS (500, 1000 mg/kg) on CCl₄-induced hepatotoxicity, serum ALT, AST, ALP, total protein (TP), conjugated bilirubin (CBIL), and total bilirubin (TBIL) levels in rats were determined [Table 3]. Hepatocellular toxicity induced by CCl₄ significantly ($p < 0.05-0.001$) increased ALT (102%), AST (58%), ALP (27%), and TBIL (62%) and significantly ($p < 0.001$) decreased TP (54%) when compared to the negative control.

The percentage hepato-protection calculated for SML extracts at 500 and 1000 mg/kg was for ALT: 7% and 32%; AST: 54% and 91%; CBIL: 75% and 88%; TBIL: 62% and 41%, respectively, when compared to the CCl₄ treated group. For SMS at 500 and 1000 mg/kg the change of ALT was 4% and 26%; AST: 47% and 83%; CBIL: 8% and 31%; and TBIL: 59% and 55%, respectively, when compared to the CCl₄ treated group. The percentage reduction of TP (54%) was significant ($p < 0.001$) following intoxication with CCl₄ but pre-treatment with plant extracts increased the percentage hepato-protection for TP. SML at 500 and 1000 mg/kg increased TP by 97% and 116% while SMS at 500 and 1000 mg/kg triggered a 111% and 119% increase, respectively, compared to the CCl₄ treated group. Silymarin (100 mg/kg), a known antioxidant, significantly ($p < 0.001$) increased the level of TP and significantly ($p < 0.05-0.001$) decreased the levels of ALT, AST, ALP, CBIL, and TBIL by 116%, 22%, 90%, 92%, 22%, 19%, and 36% respectively, compared to the CCl₄ intoxicated group.

Histopathological analysis

The effects of SML and SMS at 500 and 1000 mg/kg, and silymarin at 100 mg/kg on liver histology of CCl₄-intoxicated rats are presented in Figure 2. The section of the control rat's liver shows normal sinusoidal spaces with a non-congested portal tract and differentiated blood vessels and viable hepatocytes [Figure 2A]. Liver sections of rats intoxicated with CCl₄ indicated marked extensive necrosis, fatty accumulation, ballooning degeneration, gross mononuclear infiltration, and loss of cellular boundaries of the hepatocytes [Figure 2B]. Pre-treatment with SML at 500 mg/kg showed extensive amelioration of the histopathological architecture truncated by CCl₄ intoxication. However, prominent micro-vesicles with degenerating lipid cells (lipoid necrosis) were still present suggesting that hepatocyte insult (by CCl₄) was not yet fully resolved at this dose of SML [Figure 2C]. Following pre-treatment with SML at 1000 mg/kg liver tissue

with areas of fibrosis and localized areas of mild necrosis were still present, indicating that SML is only partially hepato-protective at this dosage [Figure 2D]. Hepatocytes of rats treated with 500 mg/kg SMS showed micro-vesicles and hepatocytes with hyperchromatic nuclei indicating that this dose is not fully hepato-protective [Figure 2E]. SMS at 1000 mg/kg showed infiltration of inflammatory cells mostly neutrophils along the portal tract and abundant mitotic bodies of the hepatocytes, indicating cell regeneration and hepato-protection. After pre-treatment with Silymarin at 100 mg/kg, liver tissue displayed a localized inflammatory response, and areas of fibrosis admixed with mitotic bodies; demonstrating healing by fibrosis, indicative of hepato-protection.

Effect of SML and SMS on hepatic antioxidants and enzyme markers

The levels of the liver antioxidant GSH, and enzymes CAT and SOD were decreased by 48%, 59%, and 30%, respectively, and TBARS were increased by 67% following intoxication with CCl₄ [Figure 3]. By contrast, GSH levels were increased significantly ($p < 0.05$) by 42% with pre-treatment with SML (1000 mg/kg), significantly ($p < 0.01$) by 50% after pre-treatment with SMS (1000 mg/kg), and significantly ($p < 0.001$) by 74% after pre-treatment with silymarin (100 mg/kg). Similarly, both CAT and SOD enzyme levels increased significantly ($p < 0.05$) by 28% and 18% after SML (1000 mg/kg), and were significantly ($p < 0.05$) increased by 41% and 20% after SMS (1000 mg/kg) pre-treatment, respectively.

DISCUSSION

Environmental chemical-induced hepatotoxicity is a major public health concern. Natural antioxidants ameliorate the effects of free-radical induced oxidative stress. The effects of SML and SMS on the weights of rats, their hematological indices, hepatic enzymes, and the hepatic antioxidant system were examined to reveal whether biomolecules present in these plants could offer hepato-protection from CCl₄-induced cellular insult.

Animals treated with SML showed no significant changes in body weights but those treated with SMS revealed a dose-dependent but not significant increase in body weight. An increase of the body weights of laboratory animals following a sub-acute toxicity study of the aqueous extract of *Enantia chlorantha* has been published^[44]. However, in contrast a decrease of body weights in rats following administration of an ethanolic stem-bark extract of the same plant has also been reported.^[45] Body weight changes may provide an indicator of drug effects, and have been used by others for assessment of responses to *Spondias mombin* drug therapy.^[33] This observed effect on body weights of the animals treated with the stem extract may be mediated through drug effects on the appetite centre within the hypothalamus. The increase in body weights we observed corroborates earlier reports of *Spondias mombin*-induced reduction of weight and appetite.^[33]

Neither the toxicant, CCl₄, nor the assessed hepato-protective agents, induced significant changes to hematological parameters suggesting no acute adverse effects on hematopoiesis. Decreased levels in platelet counts have been implicated in the severity of liver cirrhosis.^[46] However, platelets up regulation was observed only with the lower dose of SML but this effect was not significant. The comparative ability of methanolic extracts (500 and 1000 mg/kg⁻¹) of SML and SMS on hepatic lipid accumulation in fatty liver diseases and resolution of acute intoxication were examined. Following pre-treatment of rats with SML and SMS for 7 days before challenging with toxic insult, the alterations observed in the molecular architecture of the hepatocytes after CCl₄ were partially restored. This damage to the histopathological architecture of hepatocytes by necrosis and membrane lipid peroxidation are common aetiology mediated by CCl₄-induced liver damage.^[47-48]

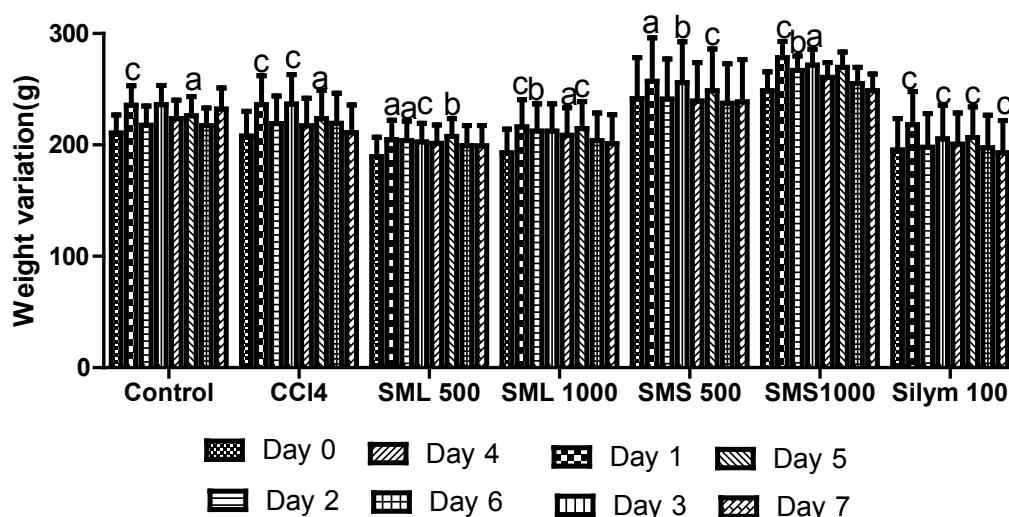


Figure 1: The effect of CCl_4 , SML and SMS on rats' weight through the course of experiment. Regarding to the effect of time, Two-way ANOVA results showed that there were significant changes in the weight of the rats of each group through the course of the experiment (0-7 days) (p -value <0.0001). However, considering the effect of treatments, Two-way ANOVA showed no significant ($p=0.506$) change among the studied groups when compared to CCl_4 treated group. Bonferroni post-test showed the differences between the weights of the rats of each group in the different days in comparison to day 0 (before the experiments). Data is shown as $M \pm SD$. (a) Means $p<0.05$, (b) means $p<0.01$ while (c) means $p<0.001$.

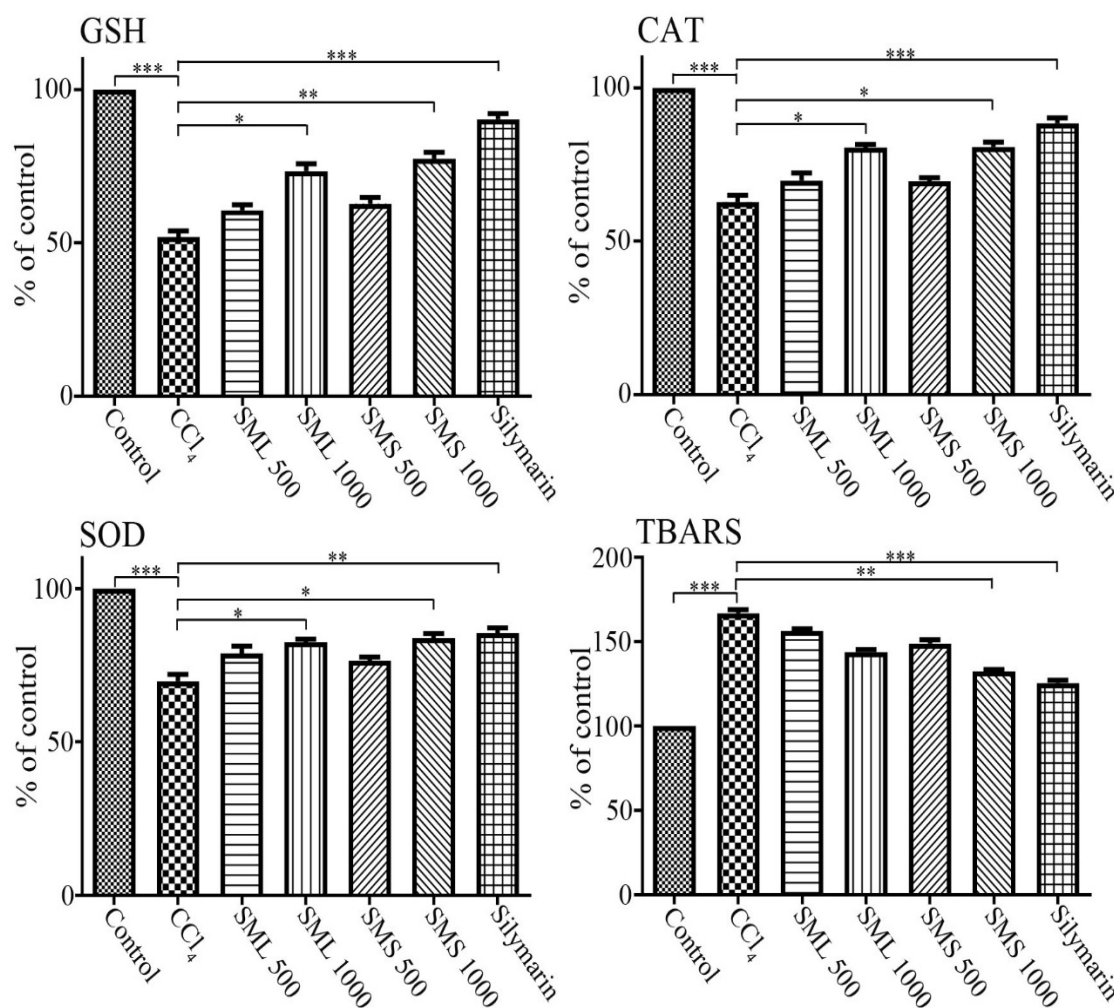


Figure 2: Effects of SML and SMS extract on markers of oxidative stress. Oxidative stress markers (GST, CAT, SOD and TBARS) were measured from homogenised liver samples. Histograms are results displayed relative to CCl_4 treated control values, with significant changes from Controls marked with asterisks. For significance: * $p<0.05$; ** $p<0.01$; *** $p<0.001$. GST: glutathione; CAT: catalase; SOD: Sodium dismutase; TBARS: Thiobarbituric acid reactive substances.

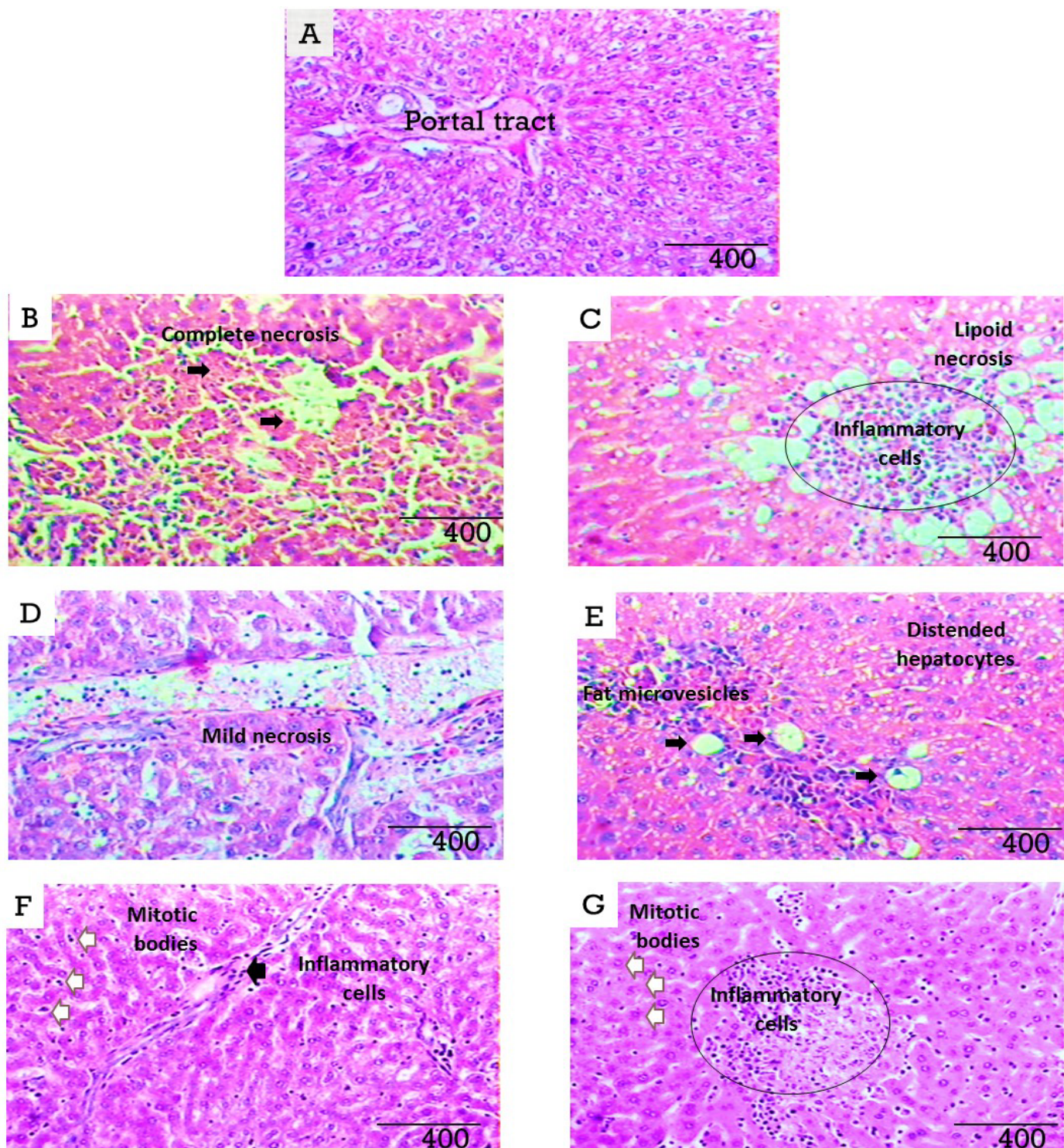


Figure 3: Photomicrograph of liver sections of rats H and E \times 400. **A:** micrograph of hepatocytes of rat treated with 0.2 ml/kg distil water showing normal liver tissues with prominent hepatocytes, hepatic artery, portal tract, normal blood vessel and hepatocytes. **B:** micrograph of hepatocyte from rodent treated with CCL_4 shows marked distortion of hepatocytes morphology with areas of complete necrosis denoting CCL_4 administered is grossly hepatotoxic at the concentration and route of administration. **C:** micrograph of hepatocyte of rat treated with CCL_4 plus 500 mg/kg of SML shows hepatocytes tissue with prominent micro vesicles with degenerating lipid cells (lipoid necrosis) revealing that hepatocytes injury due to CCL_4 not fully unresolved at this dose of SML. **D:** micrograph of hepatocyte of rat treated with CCL_4 plus 1000 mg/kg methanolic SML showing a liver tissue with areas of fibrosis and localized area of mild necrosis indicating that SML is hepatoprotective at this particular dosage; **E:** micrograph of hepatocytes of rats treated with CCL_4 plus 500 mg/kg of methanolic SMS showing liver tissue with micro vesicles and hepatocytes with hyper chromatic nuclei; dosage not quite hepatoprotective; **F:** micrograph of hepatocyte from rat treated with CCL_4 plus 1000 mg/kg methanolic SMS showing infiltration of inflammatory cells mostly neutrophils along the portal tract and abundant mitotic bodies, indicating cell regeneration therefore substance is hepatoprotective at this dosage; **G:** micrograph of liver section from hepatocyte treated standard drug, silymarin (100 mg/kg) showing liver tissue with localized inflammatory response and also areas of fibrosis admixed mitotic bodies; indicating healing by fibrosis and substance is hepatoprotective.

The toxicant CCl_4 induced hepatotoxicity with significantly increased levels of AST, ALT, ALP, CBIL and TBIL. Upregulation in the levels of serum aminotransferases are surrogates of liver injury. Hepatic damage caused after CCl_4 treatment triggers both an increase in serum transaminases and alkaline phosphatase activity.^[49] Both doses of plant extracts were significant ($p < 0.01$ - 0.001) at mediating effective reduction of CBIL, TBIL and ALP compared to the positive control (CCl_4). The SMS extract (1000 mg/kg) provided higher hepato-protectivity than the SML for the reduction of CBIL, TBIL, and ALP. The higher doses of both extracts produced higher hepato-protective effects when assayed for ALT and AST levels than the lower doses, with SMS more hepato-protective than SML. The level of TP was significantly ($p < 0.001$) reduced following acute induction of hepatotoxicity with CCl_4 , but pre-treatment with SML and SMS significantly ($p < 0.001$) normalized the TP level compared with the intoxicated rats.

The mechanism of CCl_4 intoxication of laboratory animals includes reactive oxygen species (ROS) generation and depletion of glutathione to induce oxidative stress.^[50] Administration of CCl_4 generates the free radical $\text{CCl}_3\cdot$ which causes hepatic damage due to the activation of the NADPH-Cyt P₄₅₀ system of liver endoplasmic reticulum^[51] leading to generation of the more reactive radical, trichloromethyl peroxy ($\text{CCl}_2\text{O}_2\cdot$) which provokes lipid peroxidation, disruption of Ca^{2+} homeostasis and apoptosis.^[52] These functional and morphologic changes in the cellular membrane and death of hepatocytes results in leakage of hepatic enzymes. The oxidation of fatty acids by free trichloromethyl radical ($\text{CCl}_3\cdot$) liberates lipid peroxides.^[51] These free radicals are also a likely inducer of oxidative stress within a milieu deficient in antioxidants.

The mechanisms of defense against free radicals includes mobilization of radical scavengers and chain terminators such as vitamins C and E, and antioxidants such as glutathione (GSH), and redox regulatory enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx). Therefore the tannins, saponins, alkaloids, flavonoids, phenols and ascorbic acid reported to be abundant within the SML extracts of *Spondias mombin*^[34] might play a role in this regard. This necessitated an evaluation of the effect of SML and SMS on GSH levels, CAT and SOD activities, and the levels of TBARS, markers of lipid peroxidation. Our results demonstrate that the marked elevation in TBARS induced by CCl_4 was reduced by SML and SMS significantly and dose dependently.

Depletion in liver GSH is related frequently to hepatic fatty infiltration in different experimental models^[16,17,35], and the pathophysiological consequences of GSH decline have been widely investigated. The reduction of serum GSH accompanying CCl_4 intoxication may relate to free radical generation. Hence the pre-treatments with SML and SMS that mediated increased GSH levels may well be a reflection of plant chemicals that scavenge free radicals. Indeed, serum GSH level is a sensitive biomarker of antioxidant status playing a pivotal defensive role against oxidative insults as an endogenous scavenger of free radicals.^[54,55] The five-fold decreased GSH levels in CCl_4 intoxicated rats was significantly countered, and in a dose-dependent manner, by pre-treatment with SML and SMS. This implies that SML and SMS could improve antioxidant potential in the circulation by elevating GSH concentration thereby depressing oxidative-induced damage. Certainly the liver is reported to maintain GSH even under the condition of elevated lipid peroxidation due to a supportive and compensatory mechanism.^[55,56]

The role of SOD as an antioxidant is to convert superoxide to hydrogen peroxide thereby protecting against the pervasive harmful effects of superoxide. The increased activity of SOD after SML or SMS pre-treatment compared to the decrease observed with CCl_4 intoxicated rats, might also be in part responsible for the plant extracts hepato-

protective mechanism. The elevation of CAT activity detected after the SML and SMS pre-treatments may be due to increased production of hydrogen peroxide, since CAT is a H_2O_2 scavenger. Collectively, we hypothesize that the elevation of SOD and CAT activities and GSH levels in *Spondias mombin* treated groups will augment the endogenous antioxidant system, via biomolecules directly present within SML and SMS extracts.

The restorative effects upon liver cytoarchitecture of silymarin after CCl_4 treatment may leave the liver with scar tissue, but extensive fibrosis was not observed with SMS at 1000 mg/kg. Hence cellular regeneration may have arisen via activation of liver stem cells. The mechanistic intervention of amelioration of CCl_4 -induced damage by SML and SMS might also be due to membrane stabilization to prevent leakage of cellular contents, in keeping with other hepato-protective studies including the use of *Vernonia amygdalina*,^[57] *Rumex crispus*,^[58] *Chrysophyllum albidum*^[53] as well as *Ocimum gratissimum* and *Spondias mombin*.^[35]

CONCLUSION

Both SML and SMS extracts are thought to display hepato-protective effects through a stabilizing effect on hepatocyte cell membranes, promoting repair of injured hepatic tissues, enhancement of radical scavenging effects, and augmentation of antioxidant systems limiting oxidative insults. These hepato-protective effects provide the premise for further evaluation of the promising therapeutic potential of *Spondias mombin* to counter live damage or disease that is mediated by free radicals. A protective effect of plant extracts against CCl_4 -induced liver damage has been ascribed to the presence of endogenous constituents including flavonoids, tannins, triterpenoids and alkaloids.^[59,60] Flavonoids represent the most common and extensively distributed groups of plant polyphenols and do serve as free-radical scavengers and super antioxidants to prevent oxidative cell damage.^[61] The presence of flavonoids and saponins in the leaves of SML has been reported.^[62] *Spondias mombin* derived antioxidants, particularly polyphenols, could contribute to the antioxidant activities and hepato-protection.^[63,64] However, further investigation is required to isolate the bioactive agents and further characterize the biochemical mechanisms responsible for antioxidant and hepato-protective activities of SML and SMS extracts. This work is ongoing in our laboratory and may well lead to the identification of a substance or substances of potential clinical benefit to counter liver damage and diseases.

Disclosure

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